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Interfacial Properties and Critical Micelle Concentration of Lysophospholipids†

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ABSTRACT: The critical micelle concentration (cmc) of several lysophospholipids and of a lysophospholipid analogue was determined from surface tension measurements using the maximum bubble pressure method and/or ^{31}P NMR. The use of the maximum bubble pressure method has now been extended to micromolar concentrations of surfactant, and the experimental parameters that effect its use have been explored. Surface activity was found to vary with changes in the chain length and in the headgroup polarity of the lysophospholipid. The cmc's for 1-decanoyl-, 1-dodecanoyl-, 1-tetradecanoyl-, and 1-hexadecanoyl-*sn*-glycero-3-phosphocholine are 7.0, 0.70, 0.070, and 0.007 mM, respectively. The cmc's for 1-decanoyl- and 1-dodecanoyl-*sn*-glycero-3-phosphoethanolamine are 4.4 and 0.33 mM, respectively. The cmc for dodecylphosphocholine, a lysophospholipid analogue, was found to be 1.1 mM. The cmc's for 1-tetradecanoyl- and 1-hexadecanoyl-*sn*-glycero-3-phosphoglycerol were found to be 3.0 and 0.60 mM, respectively, in pure water. In 0.1 M Tris-HCl (pH = 8.0), their cmc's are 0.16 and 0.018 mM, respectively. Surface tension and adsorption density values determined at the cmc are reported for each compound. The relationship of dynamic surface tension and lipid purity is discussed. These studies provide information about the micellization and interfacial properties of several biologically important lysophospholipids.

Lysophospholipids comprise a very important subclass of phospholipids that exhibit unique physical and biological properties not found in their parent phospholipids [for review, see Stafford and Dennis (1988)]. Unlike diacylphospholipids, which naturally form bilayers, lysophospholipids are more soluble, and they can disrupt the structure of biological membranes. Although lysophospholipids make up less than 5 mol % of the total phospholipids in a normal cell, higher lysophospholipid concentrations are associated with certain disease states, cell fusion and cell lysis [for review, see Weltzien (1979)]. Very little information has been available on the physical properties of lysophospholipids, other than 1-hexadecanoyllysophosphatidylcholine (Stafford & Dennis, 1988). Only recently have the physical properties of phospholipids and lysophospholipids such as PE[1] (Yeagle & Sen, 1986), lyso-PE (Tilcock et al., 1986), and PG (Macdonald & Seelig, 1987; Seelig et al., 1987; Eklund et al., 1987) been studied in relation to cellular metabolism and membrane structure.

It is well-known that increased phospholipase activity can occur upon the formation of a lipid–water interface [for review, see Dennis (1983)]. In our studies of human amnionic lysophospholipase (Jarvis et al., 1984) and of a lysophospholipase from the macrophage-like cell line P388D_1 (Zhang & Dennis, 1988), we originally observed that the enzymatic activity of these enzymes toward micellar lipids was apparently much greater than their enzymatic activity toward monomeric lipids. In order to study the relationship between the formation of an interface and enzymatic activity, it is necessary to know the cmc of each lipid substrate. cmc's have been reported for

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[1] Abbreviations: cmc, critical micelle concentration; lyso-PC, 1-acyl-*sn*-glycero-3-phosphocholine; lyso-PE, 1-acyl-*sn*-glycero-3-phosphoethanolamine; lyso-PG, 1-acyl-*sn*-glycero-3-phosphoglycerol; PG, 1,2-diacyl-*sn*-glycero-3-phosphoglycerol; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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only a few lyso-PC's of specific chain lengths, and the values for any given one have varied by as much as 50% (Haberland & Reynolds, 1975; van Dam Mieras et al., 1975; Kramp et al., 1984; Nakagaki et al., 1986). Furthermore, a report by Hayashi et al. (1972) on the cmc's of several alkylphosphocholines has been referred to incorrectly as giving the cmc's for several lyso-PC's (Haberland & Reynolds, 1975; Tanford, 1981). Thus, very little reliable information on the cmc's of lyso-PC's is available, let alone information on other polar headgroups.

Previous experiments have demonstrated that the ^{31}P NMR chemical shift of dihexanoyl-PC moves upfield upon its incorporation into micelles both in the presence and in the absence of the surfactant Triton X-100 (Plückthun & Dennis, 1981), making this a suitable method to study the micellization of relatively short chain phospholipids. We have now utilized this method to determine the cmc's of some lyso-PC's. However, the ^{31}P NMR method is limited to measuring cmc's that are greater than about 100 μM and thus is not sensitive enough to measure cmc's of longer chain lipids. Therefore, a second, more sensitive, method for determining cmc's was needed. The one adopted is based on the measurement of surface tension, which shows a change of slope at the cmc of the surface tension vs log concentration curve. The surface tension was measured by using the maximum bubble pressure method with an apparatus developed by Mysels (1984, 1986a). The apparatus permits the measurement of surface tension as a function of time, i.e., of dynamic surface tension over a time period of a fraction of a second to hours. We have now explored the experimental parameters that effect its use. Obtaining pure lysophospholipids is difficult, and this method permits both the detection of impurities and the determination of the cmc despite their presence (Roda et al., 1983; Mysels, 1984, 1986b). The apparatus has been used previously to determine cmc's in the millimolar range of bile acids (Mysels, 1984) and of dihexanoyl-PE (Plückthun et al., 1985). We have now adapted it to measurements in the micromolar range and employed it to determine the cmc's of a number of lyso-PC's, lyso-PE's, and lyso-PG's. The results reported herein are essential to the interpretation of enzymatic studies on a macrophage lysophospholipase (Zhang & Dennis, 1988) and other enzymes acting on lysophospholipids (R. E. Stafford, Y. Zhang, and E. A. Dennis, manuscript in preparation).

Experimental Procedures

Materials. Phospholipids were purchased from Avanti Polar Lipids. Triton X-100 was obtained from Rohm and Haas. Dodecylphosphocholine was a gift from Dr. H. S. Hendrickson, St. Olafs College, Northfield, MN. All solvents were of Optima grade (Fisher) or Gold Label (Aldrich).

Because the surface tension method is very sensitive to surfactant contaminants, solvents used for final precipitations or chromatographic purification were further purified by standard methods (Gordon & Ford, 1972), treated with activated charcoal, and subsequently distilled under dry argon. In addition, all glassware contacting the lipids was baked at 200 °C for at least 12 h and then used immediately upon cooling. The purity of the lipids was assessed by thin-layer chromatography and by ^{1}H and ^{31}P NMR in organic solvents. All lipids produced one spot on an overloaded thin-layer chromatogram (silica gel G, Merck) in solvent I, $CHCl_3$–MeOH–H_2O (65:25:4), and solvent II, $CHCl_3$–MeOH–acetic acid–H_2O (25:15:4:2). ^{31}P NMR spectra exhibited a single phosphorus resonance for each lipid.

1-Acyl-sn-glycero-3-phosphocholine. 1-Acyllyso-PC's were purified by precipitation with cold anhydrous diethyl ether (Plückthun & Dennis, 1981). The precipitate was dried under reduced pressure. Then the lipid was dissolved in glass-distilled, deionized water and lyophilized. The final product was stored for up to 1 week in a desiccator at −20 °C. We (Plückthun & Dennis, 1982a) have previously studied migration of the fatty acyl group between the *sn*-1 and *sn*-2 positions of lyso-PC, and the equilibrium mixture was found to be approximately 9:1 1-acyl- to 2-acyllyso-PC. We assume that the lysophospholipids employed herein may contain up to 10% of the 2-acyl conformer.

1-Acyl-sn-glycero-3-phosphoglycerol. 1-Acyllyso-PG's were made by hydrolysis of 1,2-diacyl-PG by snake venom phospholipase A_2 (*Naja naja naja*) in an ether–water solvent. The diacyl-PG lipids were purchased or made by transphosphatidylation of diacyl-PC as described by Comfurius and Zwaal (1977). In general, 100 mg of diacyl-PG and 10 mol % sphingomyelin [sphingomyelin is used to activate the enzyme toward non-phosphocholine-containing lipids (Adamich et al., 1979)] were suspended in 12 mL of diethyl ether in a 250-mL round-bottom flask. To this, 37 mL of 50 mM borate buffer, pH 7.0, containing 1.5 mM $CaCl_2$ was added, followed by 0.1–0.2 mg of snake venom phospholipase A_2 (*N. naja naja*). The flask was sealed and then shaken, not stirred, vigorously at 30 °C. The reaction was usually 90% complete after 1–2 h and 99% complete in 8–10 h. After the reaction flask was cooled to room temperature, the reaction was stopped by the addition of 1 mL of 200 mM EGTA, pH 6.0. The suspension was extracted by a modification of the method of Eibl and Lands (1970) as follows: 160 mL of MeOH, 122 mL of H_2O, and 148 mL of petroleum ether were mixed with the ether–water. This was extracted twice with 480 mL of petroleum ether (bp 40–60 °C)–diethyl ether (1:1). The lyso-PG remained in the lower MeOH/H_2O phase. The MeOH was distilled off under reduced pressure at room temperature. The vacuum had to be increased very slowly to avoid excessive foaming or bumping. It took approximately 8–10 h to remove all the MeOH from the H_2O. The solution was then filtered on a Whatman G/GF filter to remove a white precipitate formed during the evaporation. The sample was then lyophilized. The yield was 80–90%.

The lyophilized lipid was dissolved in about 1 mL of $CHCl_3$–MeOH–H_2O (40:13:2). The flask was washed with an additional 0.5–1 mL of the same solvent. This was centrifuged at 3000*g* for 5 min. The supernatant was applied to a CM-52 cellulose (Whatman) column (1 × 20 cm), packed in $CHCl_3$–MeOH (1:1), and subsequently equilibrated and washed according to Comfurius and Zwaal (1977). The purified lyso-PG eluted in MeOH–$CHCl_3$ (30:70). This was dried under vacuum at room temperature. The dried lipid, under dry argon, was taken up in pure water and lyophilized. The lipid was stored dry at −20 °C. The yield was 40–60%. TLC was carried out on silica gel G giving R_f (solvent I) 0.11 and (solvent II) 0.5.

1-Acyl-sn-glycero-3-phosphoethanolamine. 1-Dodecanoyllyso-PE as purchased required no further purification. 1-Decanoyllyso-PE was obtained as a byproduct in the synthesis of 1,2-didecanoyl-PE from egg PE as described previously (Plückthun & Dennis, 1982b). 1-Decanoyllyso-PE was recovered (~200 mg from 2 g of egg PE starting material) after silicic acid chromatography following acidic detritylation of the PE product. This was further purified by preparative TLC on silica gel G60 (Merck) in $CHCl_3$–MeOH–H_2O (65:25:4). The lipid was extracted from the silica with 50 mL of $CHCl_3$–MeOH (1:1) and filtered through a Whatman G/GF glass filter. The silica was washed with an additional 150 mL

of the same solvent. The solution was dried and evaporated under vacuum. The lipid was precipitated in absolute ethanol cooled to −20 °C, followed by precipitation in cold diethyl ether–MeOH as described above.

Smaller amounts of 1-decanoyllyso-PE can be obtained by hydrolysis of didecanoyl-PE by snake venom phospholipase A_2 in the presence of 10 mol % sphingomyelin and separated on CM-52 cellulose as described for lyso-PG.

Surface Tension Measurements. The underlying principle of this method for determining cmc's is explained by Roda et al. (1983). In short, the maximum pressure at which a small bubble is stable at the end of a capillary tube submerged in a liquid is proportional to the surface tension, except for a hydrostatic correction. At higher internal pressures, the bubble becomes unstable, its volume and surface increase suddenly by several orders of magnitude, and the bubble detaches. The surface tension of the air/water interface of the bubble is dependent on the age of the surface and thus on the rate of bubble formation. This is because the surface-active molecules have to reach the surface, which requires time. In general, the higher the concentration in bulk, the less the time required to build up a surface concentration. Thus, if there are two species in solution (the surfactant and the impurities) having very different concentrations, the rapid adsorption of the more concentrated and the corresponding surface tension lowering may be readily distinguished from the slower adsorption of the more dilute one and the additional surface tension lowering due to it. As the surfactant concentration of the bulk solution increases, the equilibrium surface tension decreases. This process is described by the Gibbs equation $d\sigma = m\Gamma RT$ (d ln C), where σ is the surface tension, Γ is the adsorption density, m is the number of species that are transported to the interface, and C is the monomeric surfactant concentration (or more exactly surfactant activity). For zwitterions in pure water and anions in a buffered solution, $m = 1$, and for anions in pure water, $m = 2$. At and above the cmc, additional surfactant is incorporated into micelles, increasing the total lipid concentration but effecting only a small change in the monomer concentration resulting in little change in the surface tension. A plot of the surface tension versus the log of bulk lipid concentration will have a break in the slope at the cmc. Adsorption densities Γ were calculated from the Gibbs equation. ($d\sigma$/d ln C) was obtained from the maximum slope of the line at the cmc on a plot of surface tension versus the log of bulk lipid concentration.

The improved bubble method apparatus used in this laboratory to measure surface tension has been described previously (Mysels, 1986b). Modifications in the procedure are described below. Bubble intervals ranged from 0.4 s to 1 h. Measurements were made in volumes ranging from 2 to 100 mL at 25 °C, unless otherwise noted. Concentration changes were made by dilution of the sample. Prior to dilution, an aliquot of the lipid was removed. The lipid concentration of this aliquot was determined by using a phosphate assay described below. Buffers were made with recrystallized Tris base mixed with concentrated HCl to give the desired pH.

The adsorption of lyso-PC, lyso-PE, and lyso-PG onto an air/water interface was studied. This was done by observing the effect of stirring a solution of lysophospholipid on the rate of adsorption of the lysophospholipid onto the air/water interface. The theory and experimental details of these experiments are discussed elsewhere (Mysels & Stafford, 1989). Briefly, stirring experiments were done in a jacketed flask that contained a magnetic stir bar that was rotated at a constant speed. The effect of stirring on the maximum bubble pressure itself was eliminated by proper placement of the bubble-forming capillary. All other conditions for measuring dynamic surface tensions of the stirring solution were the same as the nonstirring solution. The rate of lysophospholipid adsorption was followed by recording the surface tension as a function of bubble interval.

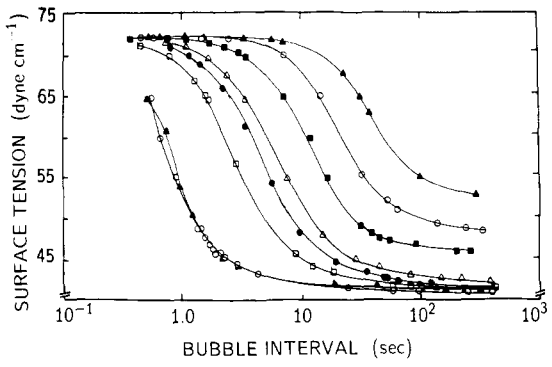

FIGURE 1: Dependence of surface tension on the bubble interval for the following concentrations of 1-tetradecanoyllyso-PC in pure water: (○) 410 μM; (▲) 160 μM; (□) 120 μM; (●) 71 μM; (△) 57 μM; (■) 41 μM; (○) 17 μM; (▲) 9.5 μM.

^{31}P NMR Measurements. For ^{31}P NMR, a stock solution of the phospholipid was prepared by dissolving a weighed sample of lipid in D_2O. All other solutions were made by dilution from the stock solution. The exact concentration of each solution was determined by phosphate analysis.

^{31}P NMR spectra were obtained at 80.99 MHz with broad-band decoupling on a 200-MHz Nicolet spectrometer at the NMR center at the University of California, San Diego. The temperature was 40.0 ± 0.5 °C. All chemical shift measurements were carried out in Wilmad 516 CC 10-mm coaxial tubes with 5% trimethyl phosphate in D_2O (v/v) as a secondary standard. All chemical shifts are reported relative to 85% phosphoric acid (upfield is negative). The chemical shift of the secondary standard was found to be +3.02 ppm. All reported chemical shifts are an average of three measurements. cmc values were obtained from plots of the ^{31}P NMR chemical shift of the lysophospholipid versus the log of the lipid concentration.

Phosphate Assay. Phospholipid concentrations were generally determined by an ammonium molybdate phosphate assay (Eaton & Dennis, 1976). For lipid concentrations of less than 100 μM, a modified malachite green/ammonium molybdate phosphate assay was employed (Hess & Derr, 1975; Lanzette et al., 1979).

Results

cmc Determination by Surface Tension. Typical measurements of the surface tension of 1-tetradecanoyllyso-PC as a function of bubble interval in pure water are shown in Figure 1. As the interface ages (longer bubble intervals), the surface tension decreases due to the increase in adsorption of surfactant. The surface tension continues to decrease with time until an equilibrium between the adsorbed surfactant and the monomer concentration is established. At very short bubble intervals, there is very little adsorption of lipid and therefore little change in surface tension, but as the bubble interval increases, the surface tension begins to decrease until it reaches a plateau which represents the surface tension at which the adsorption rate equals the desorption rate which is the equilibrium surface tension.

The cmc is obtained from the break observed in a plot of the equilibrium surface tension values as a function of the log of the lysophospholipid concentration. In Figure 2, such a plot

Table I: Critical Micelle Concentrations, Surface Tensions, and Adsorption Densities of Lysophospholipids and Analogues

lysophospholipid	surface tension[a] (dyn/cm)	critical micelle concentration (mM) bubble method	^{31}P NMR	lit.	Γ (mol cm^{-2}) $\times 10^{-10}$
1-decanoyllyso-PC	44.5	7.0	7	6.0[d]	3.1
1-dodecanoyllyso-PC	43.5	0.70	0.7		3.7
1-dodecanoyllyso-PC[c]	40.0	0.70		0.43[e]	4.0
1-tetradecanoyllyso-PC	42.3	0.070		0.043,[e] 0.063[h]	4.8
1-hexadecanoyllyso-PC	41.6	0.0070		0.007,[f] 0.0043,[e] 0.0083[h]	5.2
1-octadecanoyllyso-PC		insoluble		0.0004[e]	
1-decanoyllyso-PE	35.8	4.4			4.2
1-dodecanoyllyso-PE	32.3[b]	0.33[b]			5.2
1-tetradecanoyllyso-PE		insoluble			
1-tetradecanoyllyso-PG	49	3.0			1.0
1-tetradecanoyllyso-PG (0.1 M Tris-HCl)	46	0.16			2.4
1-hexadecanoyllyso-PG	54	0.60			1.7
1-hexadecanoyllyso-PG (0.1 M Tris-HCl)	45	0.018			2.7
dodecylphosphocholine			1.1	1.0,[g] 1.1[d]	

[a] Surface tension at the cmc. [b] Measured at 30 °C. The cmt is ~28 °C. [c] 0.14 M NaCl, 0.02 M Tris-HCl, pH 7.2. [d] van Dam Mieras et al. (1975). Wilhelmy plate method. [e] Kramp et al. (1984). Wilhelmy plate method. [f] Haberland & Reynolds (1975). Equilibrium dialysis. [g] Hayashi et al. (1972). Wilhelmy plate method. [h] Nakagaki et al. (1986). Fluorescent dye.

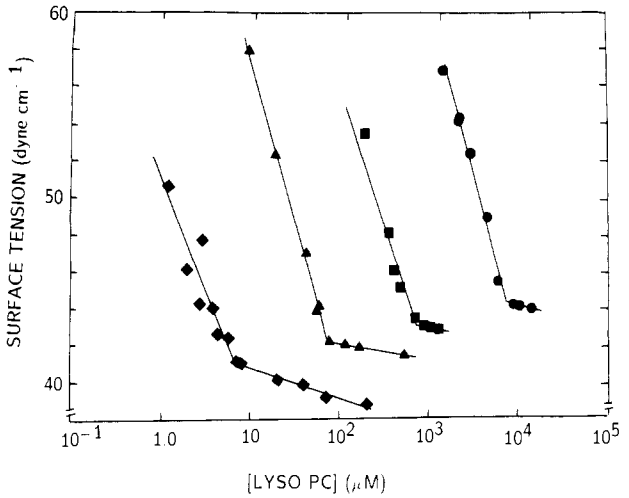

FIGURE 2: Dependence of the equilibrium surface tension on lysophospholipid concentration in pure water. The cmc is obtained from the breakpoint in each line. (◆) 1-Hexadecanoyllyso-PC; (▲) 1-tetradecanoyllyso-PC; (■) 1-dodecanoyllyso-PC; (●) 1-decanoyllyso-PC.

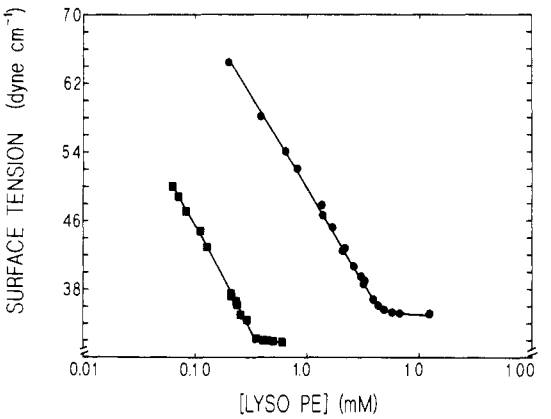

FIGURE 3: Dependence of the equilibrium surface tension on the concentration of 1-decanoyllyso-PE (●) and 1-dodecanoyllyso-PE (■) in pure water.

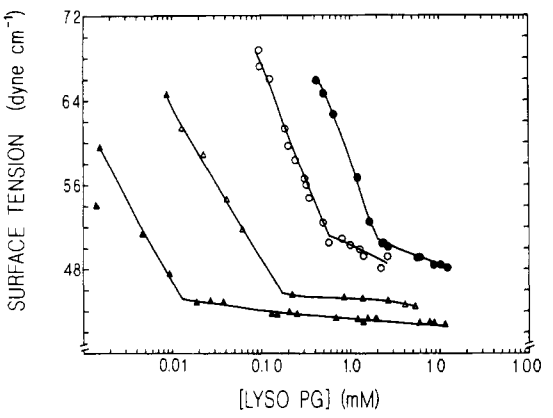

FIGURE 4: Dependence of the equilibrium surface tension on the concentration of 1-tetradecanoyllyso-PG (●) and 1-hexadecanoyllyso-PG (○) in pure water and 1-tetradecanoyllyso-PG (△) and 1-hexadecanoyllyso-PG (▲) in 0.1 M Tris-HCl buffer, pH 8.0.

for 1-tetradecanoyllyso-PC gives a cmc of 0.070 mM. The same kind of experiments with decanoyl-, dodecanoyl-, and hexadecanoyllyso-PC in pure water gave cmc's of 7.0, 0.70, and 0.0070 mM, respectively (Figure 2), as summarized in Table I. Thus, the cmc decreases by approximately a factor of 10 for each addition of two methylene groups to the acyl chain. This corresponds to a free energy of transfer (ΔG_{tr}) of one lipid molecule from water into a micelle of about −700 cal per mole of methylene group on the acyl chain of the lipid. This value is near the predicted value of −800 cal per mole for the ΔG_{tr} of amphiphilic compounds in a dilute solution (Tanford, 1981). From Figure 2, it can also be seen that the surface tension at the cmc (σ_0) decreases as the chain length is increased. Lyso-PC cmc's were unaffected by the addition of low concentrations of buffer and salt. For example, we found that the cmc of 1-hexadecanoyllyso-PC was the same in 0.14 M salt as it is in pure water (Table I).

The cmc's of two lyso-PE's were similarly determined as shown in Figure 3. For 1-dodecanoyllyso-PE, a cmc of 0.33 mM was measured at 30 °C in pure water. The cmc was measured at this temperature instead of at 25 °C because below 28.5 °C, the solution became cloudy and the lipid began to settle out of solution. The shorter chain 1-decanoyllyso-PE did not exhibit this effect, and its cmc was measured at 25 °C, also in pure water. As with the lyso-PC's, the surface tensions of the lyso-PE's decrease with decreasing chain length. However, the σ_0 of the lyso-PE's are significantly lower than those of the lyso-PC's with the corresponding chain length.

The surface tension as a function of bubble interval of lyso-PG's was measured both in 0.1 M Tris-HCl, pH 8.0, and in pure water. In buffer, the cmc's of 1-tetradecanoyl- and 1-hexadecanoyllyso-PG were 0.16 and 0.018 mM, respectively, showing a similar dependence on acyl chain length as seen with lyso-PE's and lyso-PC's (Figure 4 and Table I). The decrease

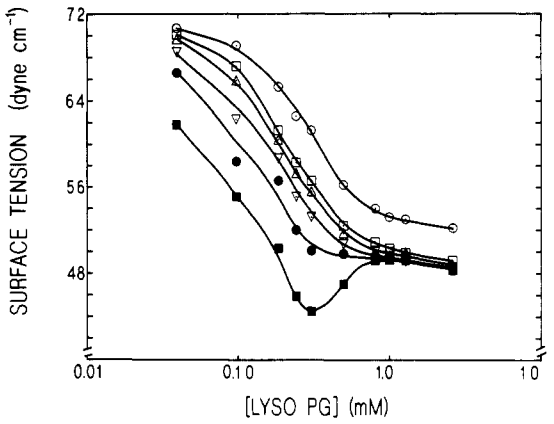

FIGURE 5: Effect of an impurity on the surface tension as a function of the concentration of 1-hexadecanoyllyso-PG at increasing bubble intervals: (○) 1 s; (□) 6 s; (△) 30 s; (▽) 100 s; (●) 200 s; (■) 1000 s.

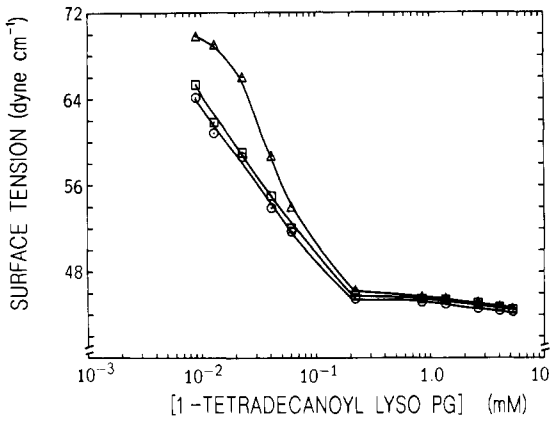

FIGURE 6: Dependence of surface tension on the concentration of 1-tetradecanoyllyso-PG in 0.1 M Tris-HCl buffer, pH 8.0, at increasing bubble intervals: (△) 10 s; (□) 100 s; (○) 1000 s.

in σ_0 with increasing lipid concentration is also similar to that of the zwitterionic lysophospholipids. The anionic lyso-PG's in pure water, however, behave very differently. The cmc's vary by only a factor of 5 when the acyl chain length is changed by two methylene groups, from 3.0 and 0.6 mM for 1-tetradecanoyllyso-PG and 1-hexadecanoyllyso-PG, respectively, and the σ_0 of the unbuffered lyso-PG is 6 dyn/cm higher than that of the buffered lyso-PG, but the σ_0 showed a similar dependence on chain length as the other headgroups.

Effect of Impurity on Surface Tension. The dynamic surface tension measurements of the unbuffered lyso-PG appear to be much more sensitive to impurities. At short bubble intervals, the surface tension reached a plateau corresponding to the equilibrium adsorption of lyso-PG, and then at longer times a second plateau occurs. This second plateau was later shown to correspond to the equilibrium of an impurity adsorbing to the interface. The time-dependent effect of the impurity on surface tension with hexadecanoyllyso-PG is demonstrated in Figure 5. For comparison, in Figure 6, the surface tension as a function of the concentration of 1-tetradecanoyllyso-PG in 0.1 M Tris-HCl, pH 8.0, which did not show the effect of an impurity, is plotted for several bubble intervals. For the hexadecanoyllyso-PG, as the bubble interval increases, the surface tension always decreases, but as concentration increases, a minimum forms in the curve in the region of the cmc. This is a clear indication of the presence of a surface effect due to an impurity as will be discussed later. We found that the contamination is due to a residual amount of fatty acid (less than 0.1%) probably from the nonenzymatic

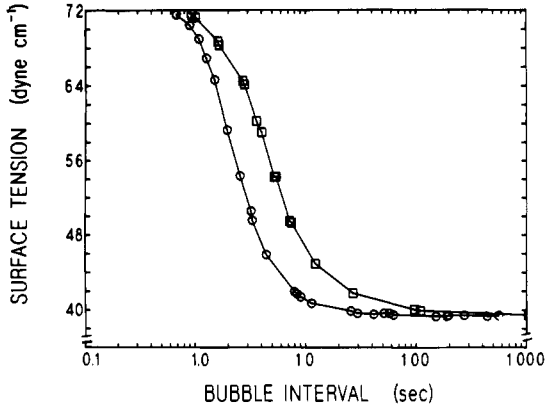

FIGURE 7: Effect of stirring on the dependence of surface tension on the age of the bubble of 220 μM 1-tetradecanoyllyso-PC: (□) no stirring; (○) 5 rps.

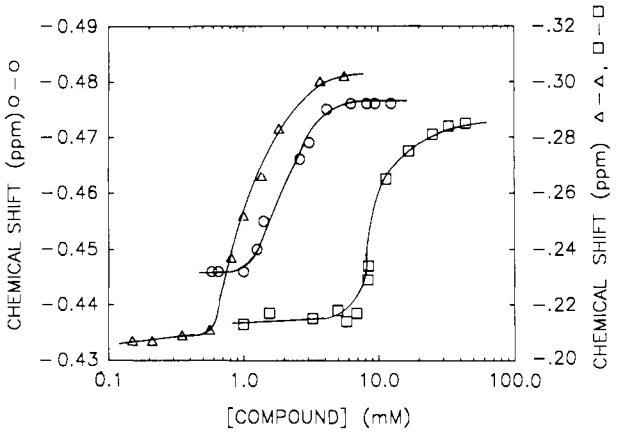

FIGURE 8: Dependence of the ^{31}P chemical shift of (□) 1-decanoyllyso-PC, (△) 1-dodecanoyllyso-PC, and (○) dodecylphosphocholine on the change in its concentration.

hydrolysis of the lysophospholipid and is at a level that is not easily detected by other techniques (see Discussion).

Stirring of solutions during surface tension measurements decreased the time for a given level of adsorption by about 50% for 1-tetradecanoyllyso-PC, as shown in Figure 7. We also found that a change in stirring rate from 0 to 5 rps decreased the adsorption time of 1-hexadecanoyllyso-PG by about 50% and decreased the adsorption time of 1-dodecanoyllyso-PE by 80–90%. This increased rate of adsorption due to increased stirring of the solution indicates that the adsorption of these lysophospholipids is largely or mainly diffusion controlled.

cmc Determination by ^{31}P NMR. Figure 8 shows the change in the ^{31}P NMR chemical shift of 1-decanoyllyso-PC and 1-dodecanoyllyso-PC in D_2O. The signal remains a sharp singlet and changes only in position upon change in concentration (Plückthun & Dennis, 1982b). Therefore, the exchange of monomers between the bulk solution and micelles is rapid compared to the NMR time scale. The chemical shift of the phosphorus begins to change at the cmc of the lipid due to a change in the average environment of the headgroup. When this change in chemical shift is plotted as a function of lipid concentration, a cmc can be estimated by drawing a line through the points corresponding to the monomeric chemical shift and a line through the points corresponding to the monomer/micelle chemical shift. For 1-decanoyllyso-PC in D_2O, the cmc was estimated from the plot in Figure 8 to be 7 mM and for 1-dodecanoyllyso-PC to be 0.7 mM. This method gives the same cmc's for these lyso-PC's as was measured by the surface tension method. The cmc of the dodecylphosphocholine using this method is 1.1 mM.

Critical micelle concentrations measured by the surface tension method and ^{31}P NMR are compared to literature values in Table I. The literature values were obtained by various methods, which are annotated in the table footnotes and are considered under Discussion. The surface tensions (σ_0) obtained at the cmc of each lipid are also reported in the table. Adsorption densities (Γ) for each lysophospholipid are presented as well. Because of the uncertainty of the slopes from surface tension vs log C plots, these values are considered approximations of the true Γ values.

Discussion

Critical Micelle Concentration. We have reported here the cmc's of a series of lyso-PC's and for the first time the cmc's of lyso-PE's and lyso-PG's. These cmc's were measured by the accurate and sensitive maximum bubble pressure method and, where possible, corroborated by ^{31}P NMR. As seen in Table I and discussed earlier, there is quite a bit of variation in the reported values of cmc's for lyso-PC's. This disparity of values for cmc's is in fact not unusual and can often be accounted for by differences in techniques or lipid purity. An advantage of dynamic surface tension measurements is that very small amounts of impurities can be detected by observing the change in surface tension at long bubble intervals. At short bubble intervals, one can accurately measure surface tensions before a large amount of any impurity adsorbs to the interface. The reliability, advantages, and disadvantages of other techniques used to determine surface tension and cmc's have been discussed elsewhere (Mukerjee & Mysels, 1971).

We have used the surface tension and ^{31}P NMR techniques to accurately determine the lysophospholipid cmc's. The value from the surface tension measurements and that extrapolated from the ^{31}P NMR measurements agree very well with that of Haberland and Reynolds (1975) for 1-hexadecanoyllyso-PC using equilibrium dialysis. In a study of cmc's of lyso-PC on varying chain lengths using the Wilhelmy plate method, cmc values were about 40% lower than those reported here. The Wilhelmy plate method also measures surface tensions. In that study, Kramp et al. (1984) made their surface tension measurements at varying lipid concentrations in 0.14 M NaCl and 0.02 M Tris-HCl, pH 7.2. Using the same conditions, we obtained the same cmc for lyso-PC as we did without salt and therefore conclude that the different ionic strength and pH are not the source of the differing results. It is possible that their lower cmc values for the same lipid are due to a contamination of the lipid–water mixture. The consistency of the values Kramp et al. (1984) obtained indicates that the concentration of such a contaminant would have to be proportional to the lipid concentration, possibly a small amount of fatty acid from hydrolysis. It is also possible that their (Kramp et al., 1984) use of an ethanol–lipid stock solution in the cmc measurements lowered the cmc values, since it has been shown that even low ethanol concentrations can depress cmc values (Benjamin, 1966; Emerson & Holtzer, 1967).

Solubility problems prevented the measurement of cmc's of long-chain lyso-PE's, although they could be measured for lyso-PE's with $n \leq 12$. In fact, long-chain lyso-PE's do not form classical micelles (Tilcock et al., 1986) but form nonbilayer and bilayer structures depending on the acyl chain composition and temperature. The cmc of dodecanoyllyso-PE was measured at 30 °C because the solution was cloudy below 28.5 °C. On the other hand, 1-decanoyllyso-PE remained a clear solution at all concentrations measured at 25 °C. The cmc's of these lyso-PE's that are measurable are about half of those of their corresponding lyso-PC's. This headgroup effect on the cmc agrees with reported observations for 1-acyl-2-[4-(4,4-dimethyl-*N*-oxyoxazolidinyl)valeryl] analogues of phospholipids. These authors have also discussed thermodynamics related to the cmc's of different phospholipid headgroups (Marsh & King, 1986; King & Marsh, 1987). They also report the temperature dependence of the cmc of several phospholipid headgroups and have found the same dependence of cmc on chain length as we have reported for lyso-PE and lyso-PC. All their measurements were made in 50 mM buffer in contrast to our measurements in pure water.

In pure water, the dependence of the cmc of lyso-PG on chain length is different from that of the other lysophospholipids and the lyso-PG in buffer. The cmc changes by only a factor of 5, instead of 10. This difference in the Δcmc is due to a common ion effect. Whereas in the case of the anionic lyso-PG in pure water, two species, the anionic lipid and the counterion, are transported from the solution to the micelle as opposed to one in the case of the zwitterionic lipids. This effect is not seen in the buffered lyso-PG solution because in such high concentrations of buffer, it takes little energy to move the counterion to the interface, and therefore the Δcmc varies by a factor of 10 instead of 5 as is the case with the zwitterionic lipids. A change in cmc by a factor of 4 has been reported for other anionic lipids (Goddard & Benson, 1957; Herrmann, 1962). In pure water, the ΔG_{tr} for an ionic surfactant is related to the Δcmc of the relation $\Delta G = nRT(2 - p/n)$ ln cmc, where n is the number of monomers and p/n is the degree of dissociation (Mukerjee, 1962). The difference in the Δcmc for anionic surfactants compared to lyso-PG is probably due to a difference in the degree of dissociation (p/n) in the micelle.

The lyso-PG has a complicated headgroup which includes one phosphate, one ester, and three hydroxyl groups. One can deduce that the headgroup is very hydrated, but neither the hydration nor net negative charge seems to have a major effect on the rate of adsorption, since we found in the stirring experiments that the adsorption of lipid to the air/water interface was mainly diffusion controlled as was found for lyso-PC and lyso-PE. The effect of stirring on the rate of adsorption of lyso-PG and lyso-PC is probably greater than we report, but excess foaming of the stirring solution may have decreased the actual rate of mixing in the region of the capillary due to drag on the solution from the layer of foam at the top of the stirring solution. We conclude that the rate of adsorption is mainly affected by the rate at which the lipid is transported to the interface. On the other hand, the equilibrium interfacial properties, such as adsorption density (Γ) and surface tensions, of these lysophospholipids are determined by the chain length, the individual properties of each headgroup, and the bulk solution properties such as ionic strength and pH. For example, Γ for 1-tetradecanoyllyso-PC is nearly 5 times that of 1-tetradecanoyllyso-PG in pure water while Γ for 1-dodecanoyllyso-PC is only about 1.3 times less than the Γ for 1-tetradecanoyllyso-PC. There is a 1.2-fold difference in Γ between lyso-PC and lyso-PE and a still smaller difference in Γ due to changes in pH and ionic strength. Lyso-PG seems to have the largest effect on some physical properties of the interface. This demonstrates that going from lyso-PC or lyso-PE to lyso-PG has a large effect on interfacial properties and that changing the hydration and polarity of interfacial lipids causes large changes in the interface. The implications of this observation are discussed in the last section.

Surface Purity. Dynamic surface tension measurements of lyso-PG in pure water were much more sensitive to impurities in the air/water interface than the buffered lyso-PG. Consequently, the lyso-PG in pure water appears to be more contaminated, although the same lipid preparation was used

in both measurements. The time-dependent effect of the contaminant on the surface tension is illustrated in Figure 5. In this plot, the dependence of surface tension on lipid concentration is plotted at different bubble intervals. At long bubble intervals a minimum occurs. This same time-dependent effect with sodium dodecyl sulfate was observed by Addison and Hutchinson (1948) and is considered a classic example of contamination (Mukerjee & Mysels, 1971). We identified the contaminant as a fatty acid by reverse-phase and $AgNO_3$ TLC. The fatty acid was identified as a hydrolysis product of the lysophospholipid, and analysis of synthetic radiolabeled lyso-PG showed that the fatty acid made up about 0.1% of the total radioactivity. Addison and Hutchinson and others (Miles & Shedlovsky, 1944; Elworthy & Mysels, 1966) have previously reported a minimum in surface tension vs sodium dodecyl sufate concentration plots of the magnitude we observed corresponding to a contaminant concentration of less than 0.5%. The generally accepted explanation for this phenomenon is that at low concentrations of surfactant the impurity goes to the surface and lowers the surface tension of the solution. At high concentrations of surfactant, the impurity is preferentially solubilized in the micelles and thus pulled away from the surface with a resulting rise of the surface tension. Interestingly, even at short bubble intervals that show no minimum, an inflection is noted at the lipid concentration corresponding to the minimum. This may be the result of the adsorption of some of the contaminant at the air/water interface, or it may be the result of the contaminant interaction with the main surfactant in the bulk solution changing the adsorption of the latter. Thus, even at short bubble intervals, there is some measurable effect of the contaminant on the lyso-PG surface tension in pure water.

The following criteria rule out the possibility that the two plateaus observed for lyso-PG were due to an adsorption and then a second plateau corresponding to perhaps a molecular rearrangement at the interface due to the unique character of the lyso-PG headgroup. By stirring the lipid solution at various speeds, we found that both equilibria were predominantly diffusion controlled and most likely due to two different surfactants. Since the effect of the contaminant only appears at concentrations below the cmc, it is likely that the contaminant is mainly sequestered in the micelle above the cmc. Below the cmc, the contaminant is able to equilibrate with the air/water interface. In this case, one would predict that the contaminant concentration would change concomitantly with the lysophospholipid concentration. Consequently, one would expect both the first and second equilibria to shift to the right with decreasing surfactant concentration, which is not observed. This is true of the surfactant but not of the impurity. It seems therefore that the contaminating fatty acid is precipitating below the cmc of the lyso-PG forming a saturated solution. In a saturated solution the contaminant concentration would remain constant, and therefore, the second equilibrium would not change upon dilution of the lipid solution, which is what is observed.

In 0.1 M Tris-HCl, pH 8.0, a decrease in the cmc of the lyso-PG is observed. This phenomenon is quite common for anionic surfactants and especially for other anionic phospholipid analogues (Marsh & King, 1986). In addition, the salt decreases the σ_0 by about 6 dyn/cm and suppresses the contaminant effects such as were seen with the same, unbuffered lipid. The decreased surface tension suggests that the interfacial concentration (Γ) of lyso-PG has increased. If the increase in Γ were due to a common ion effect analogous to the effect on the cmc, the Gibbs equation predicts that Γ would change by a factor of 2. In fact, Γ increases by more than a factor of 2.

Lysophospholipid Effects on Membrane Bilayers. The importance of membrane lipids has been recognized as possible regulators of cellular functions not only as second messengers but also because the phase properties exhibited by lipids of various acyl and alkyl chain lengths are important in cell regulation. Recently, it has been shown that lipids, detergents, alcohol, or hydrocarbons can affect membrane lipid structure, which in turn is shown to affect lipid metabolism (Lindblom et al., 1986; Wislander et al., 1986). The lysophospholipids become more soluble, in going from lyso-PE to lyso-PC to lyso-PG. This appears to be a reflection of their increased polarity and hydration. Cevc (1987) has shown that surface polarity and interfacial hydration play an important role in controlling bilayer structure. This may provide a physical explanation for the observed membrane-perturbing effects of the presumably more polar and hydrated lysophospholipids, but much more work is needed to correlate the physical properties observed herein with their biological effects.

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Registry No. 1-Decanoyllyso-PC, 22248-63-1; 1-dodecanoyllyso-PC, 20559-18-6; 1-tetradecanoyllyso-PC, 20559-16-4; 1-hexadecanoyllyso-PC, 17364-16-8; 1-decanoyllyso-PE, 120578-21-4; 1-dodecanoyllyso-PE, 120578-22-5; 1-tetradecanoyllyso-PG, 120578-23-6; 1-hexadecanoyllyso-PG, 30227-18-0; dodecyl-PC, 29557-51-5; ditetradecanoyl-PG, 61361-72-6; dihexadecanoyl-PG, 4537-77-3.

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Steady-State Catecholamine Distribution in Chromaffin Granule Preparations: A Test of the Pump-Leak Hypothesis of General Anesthesia†

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ABSTRACT: The molecular mechanism of general anesthesia is not understood. Possible modes of action include binding at a protein site, such as a receptor or channel, or physical effects on membrane lipid properties. The pump-leak hypothesis suggests that anesthetics perturb the bilayer of synaptic vesicles, thereby increasing ionic permeability. This results in decay of proton gradients required for transport and accumulation of neurotransmitters. The subsequent loss of neurotransmitters from synaptic vesicles reduces the efficiency of synaptic transmission and results in the anesthetized state. We have determined the effects of general anesthetics on certain parameters of enzyme activity and membrane permeability relevant to the pump-leak hypothesis. We used chromaffin granules as a convenient model system and focused on clinically relevant anesthetic concentrations (ED_{50}), quantitative measurements of permeability changes, and the kinetics of gradient decay. General anesthetics at ED_{50} have little or no effect on the proton-transport ATPase activity, but do cause modest increments in proton permeability that change the catecholamine distribution in actively pumping chromaffin granule preparations. We found that pH gradients do not collapse entirely under these conditions and that only a fraction of total catecholamine is lost from the chromaffin granules. When total collapse is induced by other means, efflux of catecholamines occurs with a half-time near 30 min. These results suggest that if the pump-leak hypothesis is valid, then very small losses of catecholamines must be sufficient to induce anesthesia. We conclude that the weight of evidence favors other mechanisms, notably direct binding of anesthetics to sensitive proteins.

General anesthesia is induced by compounds as diverse as xenon, nitrous oxide, halothane, and normal alcohols up to 12 carbons in length. The effect involves partitioning of the anesthetic agent into membranes of neurons, followed by inhibition of axonal and synaptic transmission. The latter is considerably more sensitive to anesthetics (Richards, 1983), and synaptic junctions presumably represent the primary site of action.

Bangham and Mason (1980) first postulated that anesthetics inhibit synaptic transmission by causing release of catecholamines from synaptic vesicles into the nerve terminal cytosol. This "pump-leak hypothesis" is based on the observation that anesthetics increase ion permeability of lipid bilayers (Bangham et al., 1965; Johnson et al., 1973; Pang et al., 1979). Thus, when anesthetic compounds partition into synaptic

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